The Proteases of Plants (VII).

BY

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IN a previous paper upon this subject (1 ; 1905) I gave an account of experiments with malt, which led me to the conclusion that it contains two proteases, the one a peptase, peptonizing fibrin; the other an ereptase, peptolyzing albumoses and peptones. I found further that the peptase was most active in an acid medium, whether of natural acidity, or when HCl had been added to the extent of 0.1 %: the addition of HCl to 0.2 %, or of Na₂CO₃ to 1 %, arrested digestion. The ereptase digested Witte-peptone most actively in an acid medium, up to HCl 0.2 %, and somewhat less rapidly in an alkaline medium containing up to 4 % Na₂CO₃. The reaction-range of the peptase was thus determined to extend from 0.2 % HCl to 1 % Na₂CO₃, whilst that of the ereptase was left undetermined, though the limit of alkalinity had been nearly reached at 4 % Na₂CO₃.

It occurred to me subsequently that since malt has such well-marked proteolytic activity, it was highly probable that the preparations of diastase which are used for medicinal purposes would possess them also. The following is an account of some experiments made in pursuance of this idea.

MALT-DIASTASE.

The first tentative experiment was made with an old specimen of diastase that had been in the laboratory for many years: the result was such as to justify further investigation.

2 grms. were mixed with 100 c.c. distilled water: the liquid was neutral, and gave no tryptophane-reaction. 40 c.c. of it were put into each of two bottles: to the one was added 0.2 gm. fibrin, to the other 0.2 gm. of Witte-peptone, with some HCN as an antiseptic. After 24 hours' digestion in the incubator at 40° C., the fibrin remained unaltered; and the contents of the other bottle gave a faint tryptophane-reaction. 24 hours later, the fibrin still remained unaltered, but the tryptophane-reaction had become marked.

These results clearly indicate the presence of ereptase, and the probable absence of peptase.

I then obtained from Merck of Darmstadt some of his 'Maltine' and of his 'Absolute Diastase'.

Experiments with Maltine.

One gram. treated with 100 c.c. distilled water formed a somewhat viscid, neutral, liquid, which was filtered: the filtrate gave a slight turbidity on boiling and on adding HNO_3 , also a distinct xanthoproteic reaction, faint biuret-reaction, but no trace of tryptophane-reaction. 40 c.c. of the filtered liquid were put into each of two bottles, to one of which 0.2 gram. fibrin, to the other 0.2 gram. Witte-peptone, were added, with a little HCN. After being in the incubator for nearly 48 hours, the contents of the bottle to which Witte-peptone had been added gave a faint tryptophane-reaction, indicating the presence of a small quantity of ereptase: the fibrin in the other bottle underwent no apparent change in 70 hours, and the liquid then gave a trace of biuret, but no tryptophane-reaction, showing that practically no fibrin-digestion had taken place.

I thought it worth while to repeat the experiment, using a stronger extract of maltine.

10 grms. were extracted for some hours with 100 c.c. water and then filtered: the filtrate was a clear, light-brown, distinctly acid liquid; on boiling a portion of it, a considerable precipitate of protein formed and was filtered off, the filtrate giving a distinct biuret but no tryptophane reaction. 40 c.c. of the filtered extract were put into each of two bottles, to one of which 0.2 gram. of fibrin was added, to the other nothing, in order that it might serve as an experiment in autolysis. Within 24 hours the fibrin had nearly disappeared: in 42 hours it had disappeared completely; the liquid in the bottle now gave marked tryptophane-reaction. The liquid in the other bottle gave a similar tryptophane-reaction at this time.

To test further the ereptic action of maltine, 0.2 gram. of Witte-peptone was digested with 40 c.c. of a 4% extract: within 24 hours the liquid gave a marked tryptophane-reaction.

Hence it appears that maltine contains proteases capable of digesting fibrin and Witte-peptone as well as its own proteins.

Subsequent experiments were made exclusively with absolute diastase, as it was found to digest much more actively than maltine.

Experiments with Absolute Diastase.

As the general method of these experiments was similar to that of those made with maltine, it will not be necessary to give full details in every case. It will suffice to say that distilled water was always used in making the extracts; that the antiseptic was HCN; that the temperature of the incubator was about 40°C .; and that several control-experiments were made with boiled extract, always with negative result.

EXPERIMENT 1. A 1 % extract: it gave no precipitate on boiling nor on adding HNO_3 : distinct xanthoproteic reaction and distinct biuret, but no tryptophane-reaction.

40 c.c. digested 0.2 gm. of fibrin in about 70 hours, the liquid then giving distinct biuret and marked tryptophane reactions.

40 c.c., to which 0.2 gm. Witte-peptone was added, gave marked tryptophane-reaction in 21 hours.

EXPERIMENT 2. A 5 % extract. Some observations were made with the object of obtaining information as to the nature of the proteins present. A portion of the extract gave a dense precipitate on boiling: the filtrate still gave evidence of the presence of protein: it was saturated with ammonium sulphate and boiled, a dense precipitate being formed: the filtrate from this precipitate gave no further precipitate on boiling and no biuret-reaction. Hence it appears that absolute diastase contains an appreciable quantity of coagulable proteins as well as albumoses.

40 c.c. of this extract digested 0.1 gm. fibrin within 22 hours, the liquid giving strong tryptophane-reaction. An autolysis experiment, carried on simultaneously, gave evidence, by a marked tryptophane-reaction, of the digestion of the native proteins.

Having thus ascertained that watery extracts of absolute diastase can digest the native proteins, fibrin, and Witte-peptone, the investigation of the effect of variations in the reaction of the liquid upon the digestive processes was undertaken.

EXPERIMENT 3. A 5 % extract used: it was distinctly acid, and gave a biuret-reaction, but no tryptophane.

In each of 4 bottles were put 35 c.c. of the filtered extract: to No. 1 nothing was added; to No. 2, 0.2 gm. of fibrin; to No. 3, 0.2 gm. of fibrin and HCl to 0.12 %; to No. 4, 0.2 gm. of fibrin and Na_2CO_3 to about 0.6 %.

After 24 hours' digestion in the incubator the results were—No. 1, the liquid gave a slight biuret-reaction; No. 2, the fibrin had been partly digested, and the biuret-reaction was strong; No. 3, much the same as No. 2; No. 4, fibrin unaltered, biuret-reaction slight.

24 hours later, the fibrin had almost entirely disappeared in Nos. 2 and 3, and the biuret-reaction was strong; the fibrin in No. 4 had been slightly attacked, and the liquid gave a distinct biuret-reaction, as did also that in No. 1. The tryptophane-test was then applied to all the liquids: the reaction was marked in Nos. 1 and 2; strong in No. 3; distinct in No. 4.

These results show that an acid reaction of the liquid promotes both the peptonizing and the peptolyzing action of the proteases, whereas alkalinity retards both. They are confirmed, as regards peptolysis, by the following experiment with Witte-peptone:—

EXPERIMENT 4. 30 c.c. of a 5 % filtered watery extract were put into each of 4 bottles: to No. 1 nothing was added; to No. 2, 0.2 gm. Witte-peptone; to

No. 3, 0.2 gm. Witte-peptone and HCl to 0.12 % ; to No. 4, 0.2 gm. Witte-peptone and Na_2CO_3 to about 0.6 %.

After 24 hours' digestion in the incubator, the tryptophane-reactions were found to be—in No. 1, faint ; No. 2, marked ; No. 3, very strong ; No. 4, faint. 48 hours later, the only changes were that the reaction had become strong in No. 2 and distinct in No. 4.

The following are the results of an experiment without added protein, that is, of an autolysis-experiment :—

EXPERIMENT 5. A 5 % watery solution was prepared: the filtered liquid was slightly acid, and gave a good biuret-reaction, but no tryptophane-reaction.

40 c.c. were put into each of 3 bottles: to No. 1 nothing was added; to No. 2, HCl to 0.15 % ; to No. 3, Na_2CO_3 to 0.625 %.

After 20 hours in the incubator, the tryptophane-reactions were—in No. 1, faint ; in No. 2, marked ; in No. 3, none: after 45 hours they were—in Nos. 1 and 2, strong ; in No. 3, none: at this time No. 3 still gave a distinct biuret-reaction, whilst Nos. 1 and 2 did not.

The results of all these experiments agree in proving that acidity promotes, whilst alkalinity retards or arrests, the digestive action of the proteases.

So far it has been assumed, on the strength of the previous experiments with malt, that there are two proteases, a peptase and an ereptase in the diastase-preparation. It remains now to adduce some evidence to justify this assumption.

With this object in view, attempts were made to isolate the proteases by means of alcohol ; three grms. of diastase were extracted, in different experiments, with 100 c.c. of 40, 50, and 60 % alcohol ; the alcoholic extracts were evaporated to half their bulk, to get rid of most of the alcohol, and then made up with distilled water to their original volume. Digestion-experiments showed that in no case had the extract any action on fibrin, but that it acted in some cases on Witte-peptone, as shown by the tryptophane-reactions.

40 % alcohol extract gave marked reaction in 24 hours, strong in 48 hours.

50 % " " " faint " " and in 48 hours.

60 % " " " no reaction in 48 hours.

Hence it appears that the peptolytic enzyme (ereptase) can be extracted, without the peptonizing enzyme (peptase), by means of 40 % alcohol.

Having determined this point, I proceeded now to ascertain what effect extraction with alcohol had had upon the diastase. The residues after extraction with alcohol of 40, 50, and 60 % respectively, were extracted with distilled water, and the action of these watery extracts upon fibrin and

Witte-peptone was tested, with the following results:—(1) in no case did the watery extract made after previous extraction with alcohol have any digestive action on fibrin in 48 hours: (2) in all three cases Witte-peptone was digested more or less; thus the watery extract of the residue previously extracted with 40 % alcohol gave distinct tryptophane-reaction after 48 hours in the incubator: those of the residues previously treated with 50 % and with 60 % alcohol both gave a strong reaction in the same time. Thus the more ereptase is extracted by alcohol, the less there is in the residue for subsequent extraction by water. The effect of treatment with alcohol upon the peptonizing activity of diastase is remarkable: it appears to be entirely destroyed. It should be added that the duration of the treatment with alcohol was 4 hours in the case of the 40 % extract, rather longer in that of the 50 % extract, and 24 hours in the case of the 60 % extract.

The preparation of extracts which acted on Witte-peptone but not on fibrin is strong evidence of the presence of ereptase as an independent enzyme. The further question remains—is the fibrin-digesting enzyme peptase? This question can only be answered conclusively by preparing from diastase an extract which digests fibrin but is without action upon Witte-peptone. I have not yet succeeded in doing this, and cannot therefore give a definite answer at present, although, on analogy, it is probable that the enzyme in question is peptase.

TAKA-DIASTASE.

This substance is prepared commercially by Messrs. Parke, Davis and Co., to whom I am indebted for the material used in these experiments; and it is employed medicinally on account of its powerful amyloclastic action. They describe it as 'a pure ferment obtained by the cultivation of a fungus of the species *Eurotium Oryzae* upon wheat-bran'.

The mould, now usually called *Aspergillus Oryzae*, Cohn, is of special interest on account of its employment on a large scale in Japan for the preparation of the alcoholic drink known as *saké*, and of the sauce known as 'soy'. For the former purpose it is cultivated upon steamed rice with the object of converting the starch of the grain into sugar; from the mixture of rice and mould, known as *koji*, a wort is prepared which is fermented with yeast. For the latter purpose the mould is cultivated upon a mixture of boiled Soja-beans (*Glycine Soja*, Sieb. et Zucc.) and roasted wheat, forming a *koji*. When the conversion of starch into sugar has proceeded far enough, a wort is prepared in which various micro-organisms induce both alcoholic and lactic acid fermentations (see Lafar, 3, and Saito, 4).

My surmise that Taka-diastrase includes proteases as well as diastase has proved to be well founded; in fact it may be said to act as vigorously upon proteins as it does upon starch. This conclusion was communicated to Messrs. Parke, Davis and Co. a year ago.

EXPERIMENT 1. A 1.5 % solution in water forms an opalescent liquid which is distinctly acid, gives no precipitate on boiling or on adding HNO_3 , and no biuret nor tryptophane reaction; but its xanthoproteic reaction is distinct. Only a very small quantity, therefore, of protein is present.

30 c.c. of the solution were put into each of two bottles: to the one was added 0.2 grm. fibrin, to the other 0.2 grm. Witte-peptone; to each a few drops of HCN.

After 24 hours in the incubator at 37°C ., the fibrin had disappeared, and the liquid gave a marked tryptophane-reaction: the liquid in the other bottle gave a very strong tryptophane-reaction; the liquid in both bottles was now strongly acid.

These results indicate great proteolytic activity, both peptonizing and peptolyzing. The next experiment was made to ascertain in what way digestion was affected by the reaction of the medium.

EXPERIMENT 2. 40 c.c. of 1.5 % solution were put into each of 5 bottles: to each bottle were added 0.2 grm. of fibrin and 0.2 grm. of Witte-peptone, and some HCN. To No. 1 nothing further was added; to No. 2, HCl to 0.1 %; to No. 3, HCl to 0.2 %; to No. 4, citric acid to 0.5 %; to No. 5, Na_2CO_3 to 0.5 %.

After 19 hours in the incubator, the fibrin had disappeared in all the bottles; the tryptophane-reactions were—in Nos. 1 and 5, marked; in Nos. 2, 3, 4, strong.

Hence it appears that the amounts of acid and of alkali added had not materially affected fibrin-digestion, whereas the added acid had distinctly promoted the digestion of Witte-peptone.

In view of the activity of the proteases, a more dilute solution was used in the next experiment (1 %), and the process of digestion was closely watched.

EXPERIMENT 3. 40 c.c. of a 1 % solution were put into each of 2 bottles: to each were added 0.2 grm. of fibrin and 0.2 grm. of Witte-peptone, and some HCN; to the one was added HCl to 0.2 %, to the other Na_2CO_3 to 1 %.

The fibrin disappeared in the acid bottle in 3 hours, in the alkaline bottle in $4\frac{1}{2}$ hours; both liquids then gave marked tryptophane-reaction.

EXPERIMENT 4. This experiment was similar to the foregoing, except that a 0.5 % solution was used.

The fibrin disappeared in $2\frac{1}{2}$ hours in the acid bottle, and in $3\frac{1}{4}$ hours in the alkaline bottle: the tryptophane-reaction was distinct in the former, faint in the latter: 19 hours later, the tryptophane-reaction was strong in the acid bottle, distinct in the alkaline.

These experiments show that both fibrin-digestion and peptolysis are more rapid in an acid than in an alkaline medium; and further, that peptolysis proceeds more slowly than fibrin-digestion.

I may quote yet one more experiment illustrating the relation between peptolysis and the reaction of the liquid.

EXPERIMENT 5. Witte-peptone was dissolved to the extent of 0.5 % in a 0.5 % solution of the diastase: the slightly acid liquid, which filtered rather slowly, gave a trace of tryptophane-reaction, and, of course, a good biuret-reaction. 40 c.c. were put into each of 3 bottles: to No. 1 no acid or alkali was added; to No. 2, HCl to 0.2 %; to No. 3, Na_2CO_3 to 1.25 %.

After 4 hours in the incubator, the tryptophane-reaction in Nos. 1 and 3 was as at the beginning; in No. 2 it had become marked. After 24 hours' digestion the reactions were—strong in No. 1; very strong in No. 2; in No. 3 but little stronger than originally. The biuret-reaction was scarcely perceptible in Nos. 1 and 2, but was undiminished in No. 3. Similar results were obtained after 48 and 72 hours' digestion. It was noticed that the acid reaction of No. 1 increased perceptibly during the experiment.

It is thus shown conclusively that Taka-diastase contains active proteases which are especially adapted to an acid medium. The reaction-ranges for protein-digestion were not determined, but they appear to be wide: with a 0.5 % solution of the substance, fibrin-digestion took place readily between 0.4 % HCl and 1 % Na_2CO_3 ; and peptolysis within the same limits, though it was diminished by 1 % Na_2CO_3 and arrested (Expt. 5) by 1.25 % Na_2CO_3 .

Some experiments were made with crude extract of the fungus, kindly placed at my disposal by Messrs. Parke, Davis and Co.; the results did not differ materially from those of the experiments here described, which were with the substance as prepared for sale, except that the digestive action was more rapid.

The question as to whether the digestive action of Taka-diastase is due to one or to two proteases had next to be considered: here again the alcohol method was employed, with the result that it was found possible to obtain, by means of extraction with alcohol, a liquid which digested Witte-peptone but had little or no action on fibrin.

EXPERIMENT 6. 1 gm. of crude Taka-diastase was treated for a couple of hours with 100 c.c. of 50 % alcohol, and then filtered; filtration was slow. The filtrate was evaporated to half its bulk at about 37° C., to get rid of some of the alcohol, and then diluted with distilled water to 105 c.c. 35 c.c. of the liquid were put into each of 3 bottles: to No. 1, 0.2 gm. of fibrin was added; to No. 2, Witte-peptone to 0.5 %; to No. 3, Witte-peptone to 0.5 % and HCl to about 0.06 %. The contents of the bottles gave a trace of tryptophane-reaction.

After 24 hours in the incubator, the fibrin in No. 1 remained unaltered; the contents of No. 2 gave marked tryptophane-reaction, and those of No. 3 a distinct reaction. 24 hours later, the fibrin still remained unaltered in No. 1, and the tryptophane-reaction had become strong in No. 2 and marked in No. 3. 24 hours later, the fibrin in No. 1 was found to have been partly digested, and the liquid gave

distinct tryptophane-reaction; the reaction given by Nos. 2 and 3 had increased in intensity.

In the meantime the residue of the diastase on the filter had been again extracted with 80 c.c. of 25 % alcohol. 20 c.c. of this filtrate were diluted with an equal volume of water and were put into a bottle with 0.2 gm. Witte-peptone; the remaining 60 c.c. were evaporated (at 37° C.) to 40 c.c., and then water was added to 80 c.c., half of it being put into each of 2 bottles, to one of which (No. 1) 0.1 gm. fibrin was added, to the other (No. 2) 0.2 gm. of Witte-peptone.

After 24 hours in the incubator, the diluted alcoholic extract gave a distinct tryptophane-reaction; the fibrin in No. 1 was mostly digested; and the contents of No. 2 gave a marked tryptophane-reaction. 24 hours later, the tryptophane-reaction of the diluted alcoholic extract was still distinct; the fibrin had entirely disappeared in No. 1, and the liquid gave faint tryptophane-reaction; No. 2 gave a distinct reaction.

The residue on the filter, which had been already twice extracted with alcohol, was further extracted with 80 c.c. of water: it did not entirely dissolve. 40 c.c. of the watery extract were put into each of 2 bottles: to No. 1 was added 0.1 gm. fibrin; to No. 2, 0.2 gm. Witte-peptone. After 24 hours in the incubator, the fibrin in No. 1 had almost disappeared; the contents of No. 2 gave a distinct tryptophane-reaction. 24 hours later, the fibrin had completely disappeared in No. 1, and the liquid gave a faint tryptophane-reaction; the tryptophane-reaction of No. 2 had become rather stronger.

This experiment shows that it is possible, by means of rapid extraction with 50 % alcohol, to obtain a liquid which has well-marked peptolytic action but digests fibrin very slowly: that it is possible, in fact, to obtain a solution containing ereptase almost alone. This result was confirmed by other experiments in which the conditions were somewhat different. In one case the diastase was treated with 50 % alcohol for 48 hours before filtration: the resulting liquid, prepared by evaporation as in the experiment just described, digested Witte-peptone, as shown by a faint tryptophane-reaction in 24 hours which increased daily in intensity at natural acidity, whereas it failed to digest fibrin at all, even though the experiment was prolonged for six days. A similar result was obtained when stronger alcohol (63 %) was used for extraction. In both these cases the action upon Witte-peptone was much retarded, in consequence of the treatment of the substance with alcohol.

Since it was thus possible to prepare an alcoholic extract of ereptase, and since, as the last-mentioned experiment shows, the residue of Taka-diastase (unlike Malt-diastase) yielded extracts, made either with more dilute alcohol or with water, which rapidly digested fibrin and had but little action upon Witte-peptone, there was ground for the assumption that the whole of the ereptase might be extracted from a small quantity of Taka-diastase, leaving behind a residue which, on extraction with water, would yield a solution which would digest fibrin but would not act upon

Witte-peptone: a solution, in fact, containing only peptase. With this object in view, the following experiment was made:—

EXPERIMENT 7. 1 gram. of Taka-diastase was mixed with 100 c.c. of 50 % alcohol and immediately filtered: the residue on the filter was then treated with 60 c.c. 50 % alcohol, and when that had filtered off, with 50 c.c. more of the same alcohol. The residue was then treated with 80 c.c. distilled water. 40 c.c. of the watery filtrate were put into each of 2 bottles: to the one was added 0.2 gram. fibrin, to the other 0.2 gram. Witte-peptone: the liquid containing Witte-peptone gave no tryptophane-reaction.

After 24 hours in the incubator, the fibrin had disappeared in the one bottle, and the contents of the other gave no tryptophane-reaction; 24 hours later, the contents of the latter bottle gave a trace of tryptophane-reaction, which did not increase during 48 hours.

I succeeded, therefore, in the attempt to remove the ereptase, and to obtain from the residue a solution which contained only peptase. This result, together with that of Experiment 6, proves that Taka-diastase contains two distinct proteases, a peptase and an ereptase.

SUMMARY.

The foregoing experiments lead to the following conclusions:—

1. Malt-diastase (whether maltine or absolute) and Taka-diastase both contain proteases which digest fibrin and produce tryptophane from albumoses and peptones whether produced by digestion or added as Witte-peptone.

2. It is possible to extract from both of these substances, by means of alcohol of various strengths, a protease which digests Witte-peptone but has no action on fibrin: this protease is therefore ereptase.

3. On washing out all the ereptase from Taka-diastase, the residue yields, on extraction with water, a solution which digests fibrin but has no action on Witte-peptone: this solution therefore contains peptase. Such a solution has not yet been obtained from Malt-diastase.

4. Since both Taka-diastase and Malt-diastase digest fibrin more rapidly in acid than in alkaline medium; and since the enzymes are excreted, in the case of Taka-diastase by the *Aspergillus Oryzae* into the organic matter upon which it is cultivated, in the case of Malt by the Barley-embryo into the endosperm; it would seem natural to refer the peptase to the group of proteases which, in my last paper on this subject (2; 1909), I termed *Ectopeptase*.

But in the course of some quite recent and still incomplete experiments I have found that solutions of the peptase of Taka-diastase, freed from ereptase (and doubtless from other substances as well) by the alcohol method (see Experiment 7, above), digest fibrin more actively when the

liquid is neutral or alkaline (e.g. 0.6 % Na_2CO_3) than when it is acid (e.g. 0.06 % HCl): that is to say, the purified peptase behaves in such a way as to suggest that it is rather an *endopeptase* than an *ectopeptase*. The question as to its nature must therefore be left open for the present.

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